

Applicants: Samuel C. Silverstein et al.  
Serial No.: 09/658, 698  
Filed : September 8, 2000  
Page 2

**Amendments to the Specification:**

Please amend the paragraph beginning at page 37, line 19 and ending at page 37, line 22, by rewriting same to read as follows:

Experimental plan: 1. Whether murine macrophages and J774 cells present the  $\beta$ -galactosidase nona-peptide TPHPARIGL (SEQ ID. NO:1) in a Class I MHC restricted fashion when they ingest IgGrRBCg+ATP.

Please amend the paragraph beginning at page 37, line 24 and ending at page 39, line 5, by rewriting same to read as follows:

Peritoneal macrophages from Balb/c mice, or J774 cells (H-2L<sup>d</sup>), loaded with TPHPARIGL (SEQ ID. NO:1) (residues 876-884 of *E. coli*  $\beta$ -galactosidase) are lysed by the H-2L<sup>d</sup> - restricted murine cytotoxic T-cell line 0805B (CTL0805B). CTL0805B cells, kindly provided by Dr. Michael Bevan, University of Washington. IgG-rRBCg will be loaded with TPHPARIGL plus LY, or TPHPARIGL plus LY plus 5 mM ATP (e.g., IgGrRBCg+TPHPARIGL (SEQ ID. NO:1)+LY+ATP or IgGrRBCg+TPHPARIGL(SEQ ID. NO:1)+LY), washed to remove free peptide, and incubated at 37°C for 1 hour with monolayers of macrophages or of J774 cells. (Ghosts containing TPHPARIGL (SEQ ID. NO:1), LY and ATP will be used in the initial experiments to confirm that these ghosts are being ingested and that ATP in them activates P2X<sub>7</sub> receptors that allow LY into the cytoplasmic and nuclear matrices. Furthermore, as described above endogenous ATP from IgGrRBCg+TPHPARIGL (SEQ ID. NO:1)+LY will be hydrolyzed by loading these ghosts with apyrase.) Uningested IgGrRBCg will be removed by lysis as described above, the peritoneal macrophages or J774 cells will be further incubated at 37°C for varying

Applicants: Samuel C. Silverstein et al.  
Serial No.: 09/658,698  
Filed : September 8, 2000  
Page 3

time periods to allow processing of the TPHPARIGL. The macrophages or J774 cells then will be labeled with  $^{51}\text{Cr}$  and incubated with various ratios of CTL0805B cells (e.g., 10-50 CTL0805B cells per target cell) for 4 hours at 37°C, at which time the medium from these cultures will be collected, sedimented to remove detached but unlysed cells, and assayed for  $^{51}\text{Cr}$  release as a measure of cytotoxicity, as described (8). Positive controls should show that CTL0805B will lyse macrophages, wild type J774 cells, or J774-P2X<sub>7</sub> null cells that were pre-loaded incubated with high concentrations of TPHPARIGL peptide prior to incubating them with CTL0805B. CTL0805B should not lyse the following cells: 1. Macrophages or J774 cells treated with cytochalasin D to prevent ingestion of the IgGrRBCg+TPHPARIGL (SEQ ID. NO:1)+ATP or of the IgGrRBCg+TPHPARIGL (SEQ ID. NO:1). 2. J774-P2X<sub>7</sub> null cells incubated with IgGrRBCg+TPHPARIGL (SEQ ID. NO:1)+ATP or IgGrRBCg+TPHPARIGL (SEQ ID. NO:1). 3. Macrophages or J774 cells treated with Brefeldin A to prevent transport of TPHPARGL (SEQ ID. NO:1)-loaded Class I MHC proteins from the endoplasmic reticulum to the surface. 4. Macrophages or J774 cells incubated with IgGrRBCg+ATP and a scrambled peptide to which CTL0805B cells do not react. Anticipated results: CTL0805B will only kill syngeneic macrophages or wild type J774 cells that have processed IgGrRBCg+TPHPARIGL (SEQ ID. NO:1)+ATP.

Please amend the paragraph beginning at page 39, line 7 and ending at page 39, line 11, by rewriting same to read as follows:

Experimental plan: 2. Whether IgCrRBCg containing TPHPARIGL (SEQ ID. NO:1) and ATP, but not IgGrRBCg containing - TPHPARIGL (SEQ ID. NO:1) but lacking ATP, can be used to

Applicants: Samuel C. Silverstein et al.  
Serial No.: 09/658,698  
Filed : September 8, 2000  
Page 4

immunize naive mice to form CTL that lyse syngeneic macrophages or J774 cells loaded with the peptide.

Please amend the paragraph beginning at page 39, line 13 and ending at page 40, line 10, by rewriting same to read as follows:

Balb/c mice will be immunized weekly for 3-6 weeks intra-peritoneally, or subcutaneously in the neck or hind footpad with rRBCg or IgGrRBCg containing TPHPARIGL (SEQ ID. NO:1) with or without ATP. As a control, similar numbers of mice will be immunized with J774 cells incubated in TPHPARIGL (SEQ ID. NO:1)-containing buffer to load Class I MHC proteins with this peptide as described (8). For mice immunized subcutaneously, T-lymphocytes will be obtained from regional lymph nodes and spleen. For mice immunized intra-peritoneally T-lymphocytes will be obtained from spleen. Spleen and lymph node cells from immunized mice will be tested for induction of CTL against TPHPARIGL (SEQ ID. NO:1)-pulsed Balb/c macrophages (syngeneic), TPHPARIGL (SEQ ID. NO:1)-pulsed C57B1/6 macrophages (allogeneic), or J774 cells, as in 1. above, and for helper T-lymphocyte activity using  $^3\text{H}$ -thymidine incorporation or IL-2 production using X-irradiated lac-Z transfected Balb/c 3T3 cells (9).

In a second series of experiments Balb/c dendritic cells or macrophages will be allowed to ingest IgGrRBCg containing TPHPARIGL with or without ATP. Uningested IgGrRBCg+TPHPARIGL (SEQ ID. NO:1) will be lysed, and these APCs will be administered to Balb/c mice weekly for 3-6 weeks intra-peritoneally or subcutaneously. The mice then will be sacrificed and their spleen and regional lymph node cells tested for CTL activity against TPHPARIGL (SEQ ID. NO:1)-pulsed J774 cells (as described in 8), and for helper T-lymphocytes using  $^3\text{H}$ -thymidine incorporation or IL-2

Applicants: Samuel C. Silverstein et al.  
Serial No.: 09/658,698  
Filed : September 8, 2000  
Page 5

production using X-irradiated lac-Z transfected Balb/c 3T3 cells as stimulators (9).

Please amend the paragraph beginning at page 40, line 12 and ending at page 40, line 19, by rewriting same to read as follows:

Anticipated results: rRBCg+TPHPARIGL (SEQ ID. NO:1)+ATP, IgGrRBCg TPHPARIGEL (SEQ ID. NO:2)+ATP, or macrophages or dendritic cells that ingested IgGrRBCg TPHPARIGEL (SEQ ID. NO:2)+ATP will induce formation of CTLs while rRBCg+TPHPARIGEL (SEQ ID. NO:2), IgGrRBCg+TPHPARIGEL (SEQ ID. NO:2), or macrophages or dendritic cells that ingested IgGrRBCg+TPHPARIGEL (SEQ ID. NO:2) will not. In contrast, all preparation will induce activation of helper T-lymphocytes.

Please amend the paragraph beginning at page 40, line 21 and ending at page 40, line 25, by rewriting same to read as follows:

Next, after obtaining positive results in the experiments described in 2 above, P2X<sub>7</sub>-knock out mice will be obtained and it will be determined whether they are incapable of mounting a helper or CTL response to IgGrRBCg+ TPHPARIGL (SEQ ID. NO:1) +ATP.